Creatinine ELISA Kit

Item No. 502330

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GENERAL INFORMATION

Materials Supplied

Item Number	Item Name	96 wells Quantity/Size	Storage Temperature
400401	Creatinine-HRP Tracer	1 vial/1 ml	-20°C
400402	Creatinine ELISA Monoclonal Antibody	1 vial/6 ml	-20°C
400403	Creatinine ELISA Standard	1 vial/250 µg	-20°C
401703	Immunoassay Buffer C Concentrate (10X)	1 vial/10 ml	4°C
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml	RT
400074	TMB Substrate Solution	2 vials/12 ml	4°C
10011355	HRP Stop Solution	1 vial/12 ml	RT
400035	Polysorbate 20	1 vial/3 ml	RT
400008/400009	Goat Anti-Mouse IgG-Coated Plate	1 plate	4°C
400012	96-Well Cover Sheet	1 ea	RT
400040	ELISA Tracer Dye	1 ea	RT
400042	ELISA Antiserum Dye	1 ea	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical's Creatinine ELISA Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

When compared to quantification by LC/MS or GC/MS, it is not uncommon for immunoassays to report higher analyte concentrations. While LC/MS or GC/MS analyses typically measure only a single compound, antibodies used in immunoassays sometimes recognize not only the target molecule, but also structurally related molecules, including biologically relevant metabolites. In many cases, measurement of both the parent molecule and metabolites is more representative of the overall biological response than is the measurement of a short-lived parent molecule. It is the responsibility of the researcher to understand the limits of both assay systems and to interpret their data accordingly.

The stop solution provided with this kit is an acid solution. Please wear appropriate personal protective equipment (*e.g.* safety glasses, gloves, and lab coat) when using this material.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the Materials Supplied section (see page 3) and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance at 450 nm
2. An orbital microplate shaker
3. Adjustable pipettes; multichannel or repeating pipettor recommended
4. A source of ultrapure water, with a resistivity of 18.2 MΩ·cm and total organic carbon (TOC) levels of <10 ppb, is recommended. Pure water - glass-distilled or deionized - may not be acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
5. Materials used for **Sample Preparation** (see page 13)

INTRODUCTION

Background

Creatinine is a degradation product of creatine and creatine phosphate, which are involved in the creatine kinase reaction that is a source of phosphate for the conversion of ADP to ATP for energy in cells and tissues that consume ATP rapidly, such as skeletal muscle and the brain.[1] Muscle creatine and creatine phosphate are converted nonenzymatically and irreversibly at a steady rate to creatinine, which is transported to the kidney in the blood and excreted in the urine.[1,2] The amount of creatinine produced is proportional to an individual's muscle mass and can be used to normalize data in assays using urine samples.[2,3] Plasma levels of creatinine have been used as a marker of acute kidney injury, while serum and urinary levels have been used as measures of the glomerular filtration rate (GFR) to assess renal function, estimate and monitor the extent of functional renal impairment, and detect renal disease.[1-4]

About This Assay

Cayman's Creatinine ELISA Kit is a competitive assay that can be used for the quantification of creatinine in plasma, serum, and urine. The assay has a range of 0.273-35 µg/ml, with a midpoint (50% B/B_0) of 2.5-5.5 µg/ml, and a sensitivity (80% B/B_0) of approximately 1 µg/ml.

Principle Of This Assay

This assay is based on the competition between free creatinine and a creatinine-HRP conjugate (Creatinine-HRP Tracer) for a limited number of creatinine monoclonal antibody binding sites. Because the concentration of the Creatinine-HRP Tracer is held constant while the concentration of free creatinine varies, the amount of Creatinine-HRP Tracer that is able to bind to the Creatinine Monoclonal Antibody will be inversely proportional to the concentration of free creatinine in the well. This antibody-creatinine complex binds to goat anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and TMB Substrate Solution (which contains the substrate to HRP) is added to the well, followed by the HRP Stop Solution. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 450 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of Creatinine-HRP Tracer bound to the well, which is inversely proportional to the amount of free creatinine present in the well during the incubation, as described in the equation:

$$\text{Absorbance} \propto \text{[bound creatinine-HRP tracer]} \propto 1/\text{[creatinine]}$$

A schematic of this process is shown in Figure 1, on page 9.

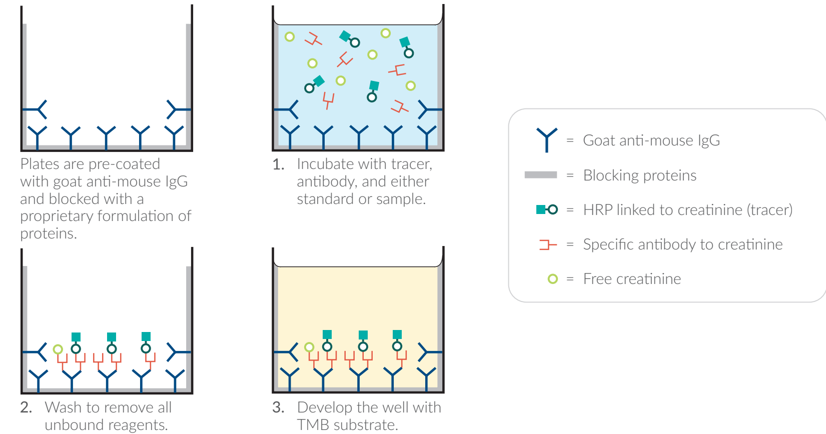


Figure 1. Schematic of the ELISA

Definition of Key Terms

Blk (Blank): background absorbance caused by TMB Substrate Solution and the HRP Stop Solution. The blank absorbance should be subtracted from the absorbance readings of all the other wells, including the non-specific binding (NSB) wells.

TA (Total Activity): total enzymatic activity of the creatinine HRP-linked tracer.

NSB (Non-Specific Binding): non-immunological binding of the tracer to the well. Even in the absence of specific antibody a very small amount of tracer still binds to the well; the NSB is a measure of this low binding.

B_0 (Maximum Binding): maximum amount of the tracer that the antibody can bind in the absence of free analyte.

$\%B/B_0$ (%Bound/Maximum Bound): ratio of the absorbance of a sample or standard well to the average absorbance of the maximum binding (B_0) wells.

Standard Curve: a plot of the $\%B/B_0$ values versus concentration of a series of wells containing various known amounts of analyte.

Cross Reactivity: numerical representation of the relative reactivity of this assay towards structurally related molecules as compared to the primary analyte of interest. Biomolecules that possess similar epitopes to the analyte can compete with the assay tracer for binding to the primary antibody. Substances that are superior to the analyte in displacing the tracer result in a cross reactivity that is greater than 100%. Substances that are inferior to the primary analyte in displacing the tracer result in a cross reactivity that is less than 100%. Cross reactivity is calculated by comparing the (50% B/B_0) value of the tested molecule to the (50% B/B_0) value of the primary analyte when each is measured in assay buffer using the following formula:

$$\%\ \text{Cross Reactivity} = \left[\frac{50\%\ B/B_0 \text{ value for the primary analyte}}{50\%\ B/B_0 \text{ value for the potential cross reactant}} \right] \times 100\%$$

Lower Limit of Detection (LLOD): the smallest measure that can be detected with reasonable certainty for a given analytical procedure. The LLOD is defined as a concentration two standard deviations higher than the mean zero value.

PRE-ASSAY PREPARATION

Buffer Preparation

Store all diluted buffers at 4°C; they will be stable for approximately two months. NOTE: It is normal for the concentrated buffers to contain crystalline salts. These will completely dissolve upon dilution with water.

1. **Immunoassay Buffer C (1X) Preparation**

   Dilute the contents of one vial of Immunoassay Buffer C Concentrate (10X) (Item No. 401703) with 90 ml of ultrapure water. Be certain to rinse the vial to remove any salts that may have precipitated.

2. **Wash Buffer (1X) Preparation**

   Dilute the contents of one vial of Wash Buffer Concentrate (400X) (Item No. 400062) with ultrapure water to a total volume of 2 L and add 1 ml of Polysorbate 20 (Item No. 400035). Smaller volumes of Wash Buffer (1X) can be prepared by diluting the Wash Buffer Concentrate (400X) 1:400 and adding 0.5 ml of Polysorbate 20 per 1 L of Wash Buffer (1X). *NOTE: Polysorbate 20 is a viscous liquid and cannot be measured by a regular pipette. A positive displacement pipette or a syringe should be used to deliver small quantities accurately.*

Sample Preparation

General Precautions

- All samples must be free of organic solvents prior to assay.
- Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be frozen.
- Samples of mouse origin may contain antibodies that interfere with the assay by binding to the goat anti-mouse plate. We recommend that all mouse samples be purified prior to use in the assay.

Testing for Interference

This assay has been validated in plasma, serum, and urine. Other sample types should be tested for interference to evaluate the need for sample purification before embarking on a large number of sample measurements. To test for interference, dilute one or two samples to obtain at least two different dilutions of each sample within the linear portion of the standard curve. If two different dilutions of the same sample show good correlation (differ by 20% or less) in the final calculated creatinine concentration, sample purification is not required. If you do not see good correlation of the different dilutions, purification is advised. Purification methods will need to be determined by the end user and tested for compatibility in the assay.

Plasma

Collect blood in vacutainers containing heparin, EDTA, or sodium citrate for plasma samples. To obtain plasma, centrifuge blood at 1,000 x g for 15 minutes at 4°C. Pipette off the top plasma layer without disturbing the white buffy layer. Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -80°C. It is recommended that plasma samples be diluted at least 1:2 into Immunoassay Buffer C (1X) prior to testing in the assay.

Serum

Collect blood in vacutainers without a coagulant for serum samples. Allow samples to clot undisturbed for 30-60 minutes at room temperature. To obtain serum, centrifuge at 1,000-2,000 x g for 15-30 minutes at 4°C. Pipette off the serum layer. Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -80°C. It is recommended that plasma samples be diluted at least 1:2 into Immunoassay Buffer C (1X) prior to testing in the assay.

Urine

Urine samples should be assayed immediately or stored at -20°C immediately after collection. Interference in urine is infrequent. Dilute urine samples with Immunoassay Buffer C (1X) to fall within the range of the standard curve.

Sample Matrix Properties

Parallelism

To assess parallelism, plasma, serum, and urine were processed as described in the Sample Preparation section (see page 13), serially diluted with Immunoassay Buffer C (1X), and evaluated using the Creatinine ELISA Kit. Measured concentrations were plotted as a function of the sample dilution. The results are shown below.

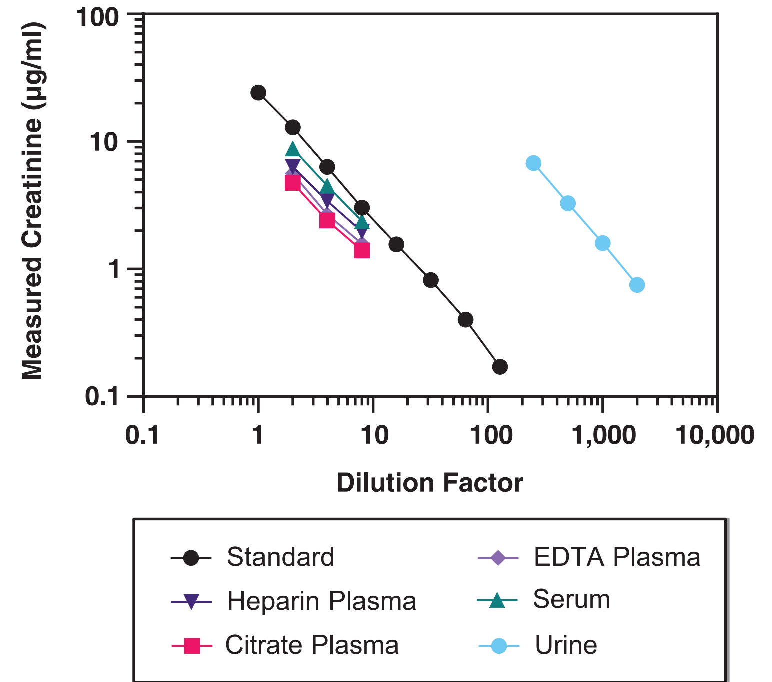


Figure 2. Parallelism of various matrices in the Creatinine ELISA

Spike and Recovery

Plasma, serum, and urine were spiked with different amounts of creatinine, processed as described in the Sample Preparation section (see page 13), serially diluted with Immunoassay Buffer C (1X), and evaluated using the Creatinine ELISA Kit. The results are shown below. The error bars represent standard deviations obtained from multiple dilutions of each sample.

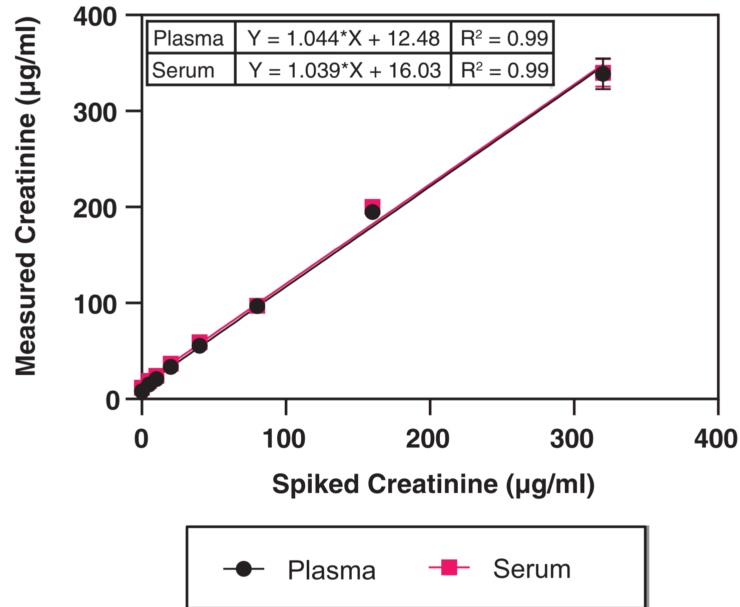


Figure 3. Spike and recovery of creatinine in plasma and serum

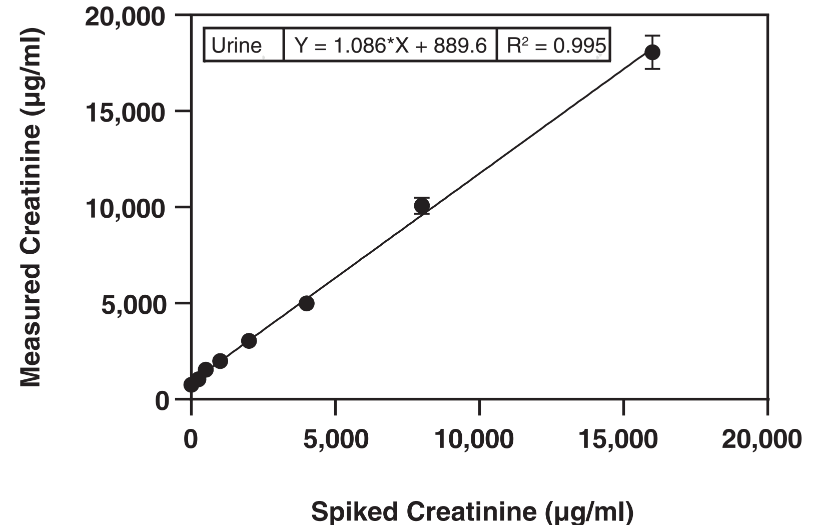


Figure 4. Spike and recovery of creatinine in urine

Linearity

Plasma and serum samples spiked with 80 µg/ml creatinine and urine samples spiked with 1,000 µg/ml creatinine were serially diluted with Immunoassay Buffer C (1X) and evaluated for linearity using the Creatinine ELISA Kit. The results are shown in Table 1 below.

Dilution Factor	Concentration (µg/ml)	Linearity (%)
Plasma		
20	92.6	100
40	99.1	107
80	98.5	106
Serum		
20	96.2	100
40	96.5	100
80	96.4	100
Urine		
400	1,999	100
800	2,024	101
1,600	1,959	98.0

Table 1. Linearity in various matrices

NOTE: Linearity has been calculated using the following formula:

%Linearity = (Observed concentration value, dilution adjusted / First observed concentration value in the dilution series, dilution adjusted)*100

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

Creatinine ELISA Standard

To prepare the standard for use in ELISA: Obtain eight clean test tubes and label them #1-8. Aliquot 860 µl of Immunoassay Buffer C (1X) to tube #1 and 500 µl Immunoassay Buffer C (1X) to tubes #2-8. Equilibrate a pipette tip by repeatedly filling and expelling the tip with the Creatinine ELISA Standard (Item No. 400403) several times. Using the equilibrated pipette tip, transfer 140 µl of the Creatinine ELISA Standard to tube #1 and mix thoroughly. Serially dilute the standard by removing 500 µl from tube #1 and placing it in tube #2; mix thoroughly. Next, remove 500 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. These diluted standards should be used within two hours.

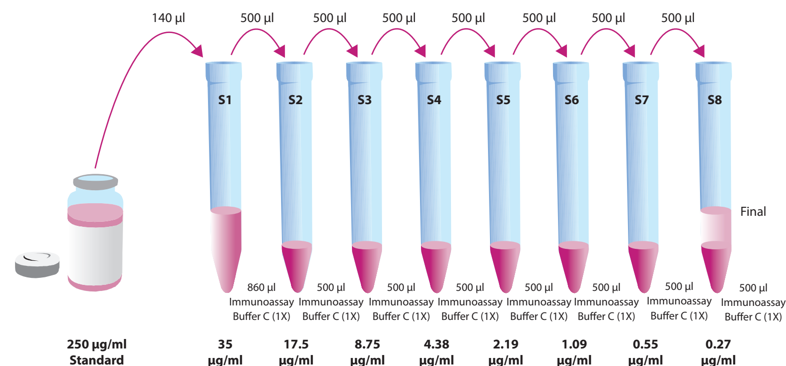


Figure 5. Preparation of the creatinine standards

Creatinine-HRP Tracer

Dilute the Creatinine-HRP Tracer (Item No. 400401) with 5 ml of Immunoassay Buffer C (1X). Store the diluted Creatinine-HRP Tracer at 4°C (***do not freeze!***). It will be stable for at least four weeks. A 20% surplus of tracer has been included to account for any incidental losses.

Tracer Dye Instructions (optional)

This dye may be added to the tracer, if desired, to aid in visualization of tracer-containing wells. Add the dye to the diluted tracer at a final dilution of 1:100 (add 60 µl of dye to 6 ml tracer). *NOTE: Do not store tracer with dye for more than three weeks at 4°C.*

Creatinine ELISA Monoclonal Antibody

The Creatinine ELISA Monoclonal Antibody (Item No. 400402) is ready to use as supplied. If all the antibody was not used at once, it can be stored at -20°C, limiting the number of the freeze/thaw cycles to two. A 20% surplus of antibody has been included to account for any incidental losses.

Antiserum Dye Instructions (optional)

This dye may be added to the antibody, if desired, to aid in visualization of antibody-containing wells. Add the dye to the antibody at a final dilution of 1:100 (add 60 µl of dye to 6 ml antibody). *NOTE: Store the antibody with dye at 4°C and use within three weeks.*

Plate Set Up

The 96-well plate(s) included with this kit must be pre-washed five times with Wash Buffer (1X) (300 µl/well) prior to use in the ELISA. *NOTE: If you do not need to use all the strips at once, place the unwashed strips back in the plate packet and store at 4°C. Be sure the packet is sealed with the desiccant inside.*

Each plate or set of strips must contain a minimum of two Blk, two NSB, and three B_0 wells, and an eight-point standard curve run in duplicate. *NOTE: Each assay must contain this minimum configuration in order to ensure accurate and reproducible results.* Each sample should be assayed at a minimum of two dilutions and each dilution should be assayed in duplicate. For statistical purposes, we recommend assaying each dilution in triplicate.

A suggested plate format is shown in Figure 6, below. The user may vary the location and type of wells present as necessary for each particular experiment. The plate format provided below has been designed to allow for easy data analysis using a convenient spreadsheet offered by Cayman (see page 24 for more details). We suggest recording the contents of each well on the template sheet provided (see page 33).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	S1	S1	1	1	1	9	9	9	17	17	17
B	Blk	S2	S2	2	2	2	10	10	10	18	18	18
C	NSB	S3	S3	3	3	3	11	11	11	19	19	19
D	NSB	S4	S4	4	4	4	12	12	12	20	20	20
E	B_0	S5	S5	5	5	5	13	13	13	21	21	21
F	B_0	S6	S6	6	6	6	14	14	14	22	22	22
G	B_0	S7	S7	7	7	7	15	15	15	23	23	23
H	TA	S8	S8	8	8	8	16	16	16	24	24	24

Blk - Blank wells
NSB - Non-Specific Binding wells
B_0 - Maximum Binding wells
TA - Total Activity well
S1-S8 - Standard wells
1-24 - Sample wells

Figure 6. Sample plate format

Performing the Assay

Pipetting Hints

- Use different tips to pipette each reagent.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

Pre-Wash the Plate

Rinse the plate (or strips to be used) five times with ~300 µl Wash Buffer (1X).

Addition of the Reagents

1. **Immunoassay Buffer C (1X)**

   Add 100 µl of Immunoassay Buffer C (1X) to NSB wells. Add 50 µl of Immunoassay Buffer C (1X) to B_0 wells.

2. **Creatinine ELISA Standard**

   Add 50 µl from tube #8 to both of the lowest standard wells (S8). Add 50 µl from tube #7 to each of the next standard wells (S7). Continue with this procedure until all the standards are aliquoted. The same pipette tip should be used to aliquot all the standards. Before pipetting each standard, be sure to equilibrate the pipette tip in that standard.

3. **Samples**

   Add 50 µl sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate (triplicate recommended).

4. **Creatinine-HRP Tracer**

   Add 50 µl to each well except the TA and Blk wells.

5. **Creatinine ELISA Monoclonal Antibody**

   Add 50 µl to each well except the TA, NSB, and Blk wells within 15 minutes of addition of the tracer.

Incubation of the Plate

Cover each plate with a 96-Well Plate Cover Sheet (Item No. 400012) and incubate 2 hours at room temperature on an orbital shaker.

Development of the Plate

1. Empty the wells and rinse five times with ~300 µl Wash Buffer (1X).
2. Add 175 µl of TMB Substrate Solution (Item No. 400074) to each well.
3. Dilute 5 µl of the previously diluted Creatinine-HRP Tracer with 95 µl of Immunoassay Buffer C (1X). Add 5 µl of this solution to the TA wells.
4. Cover the plate with the 96-Well Cover Sheet and protect from light. Optimum development is obtained by using an orbital shaker at room temperature for 30 minutes.
5. Remove the cover sheet being careful to keep TMB Substrate Solution from splashing on the cover. *NOTE: Any loss of TMB Substrate Solution will affect the absorbance readings.*
6. **DO NOT WASH THE PLATE**. Add 75 µl of HRP Stop Solution (Item No. 10011355) to each well of the plate. Blue wells should turn yellow and colorless wells should remain colorless. *NOTE: The stop solution in this kit contains an acid. Wear appropriate protection and use caution when handling this solution.*

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, *etc.*
2. Read the plate at a wavelength of 450 nm.

ANALYSIS

Many plate readers come with data reduction software that plot data automatically. Alternatively, a spreadsheet program can be used. The data should be plotted as either $\%B/B_0$ *versus* log concentration using a four-parameter logistic fit or as logit B/B_0 *versus* log concentration using a linear fit. *NOTE: Cayman has computer spreadsheets available for data analysis. Please contact Technical Service or visit our website (www.caymanchem.com/analysis/elisa) to obtain free copies of our ELISA Double or ELISA Triple Analysis tools.*

Calculations

Preparation of the Data

The following procedure is recommended for preparation of the data prior to graphical analysis.

NOTE: If the plate reader has not subtracted the absorbance readings of the blank wells from the absorbance readings of the rest of the plate, be sure to do that now.

1. Average the absorbance readings from the NSB wells.
2. Average the absorbance readings from the B_0 wells.
3. Subtract the NSB average from the B_0 average. This is the corrected B_0 or corrected maximum binding.
4. Calculate the B/B_0 (Sample or Standard Bound/Maximum Bound) for the remaining wells. To do this, subtract the average NSB absorbance from the S1 absorbance and divide by the corrected B_0 (from Step 3). Repeat for S2-S8 and all sample wells. (To obtain $\%B/B_0$ for a logistic four-parameter fit, multiply these values by 100.)

NOTE: The TA values are not used in the standard curve calculations. Rather, they are used as a diagnostic tool. Low or no absorbance from a TA well could indicate a dysfunction in the enzyme-substrate system.

Plot the Standard Curve

Plot $\%B/B_0$ for standards S1-S8 *versus* creatinine concentration using linear (y) and log (x) axes and perform a four-parameter logistic fit.

Alternative Plot - The data can also be linearized using a logit transformation. The equation for this conversion is shown below. *NOTE: Do not use $\%B/B_0$ in this calculation.*

$$\text{logit}\,(B/B_0) = \ln\,[B/B_0/(1 - B/B_0)]$$

Plot the data as logit (B/B_0) *versus* log concentrations and perform a linear regression fit.

Determine the Sample Concentration

Calculate the B/B_0 (or $\%B/B_0$) value for each sample. Determine the concentration of each sample by identifying the $\%B/B_0$ on the standard curve and reading the corresponding values on the x-axis. *NOTE: Remember to account for any dilution of the original sample concentration prior to its addition to the well. Samples with $\%B/B_0$ values greater than 80% or less than 20% should be re-assayed as they generally fall out of the linear range of the standard curve. A 20% or greater disparity between the apparent concentration of two different dilutions of the same sample indicates interference, which could be eliminated by purification.*

NOTE: If there is an error in the B_0 wells it is possible to calculate sample concentrations by plotting the absorbance values and back calculating sample absorbance off the standard curve.

Performance Characteristics

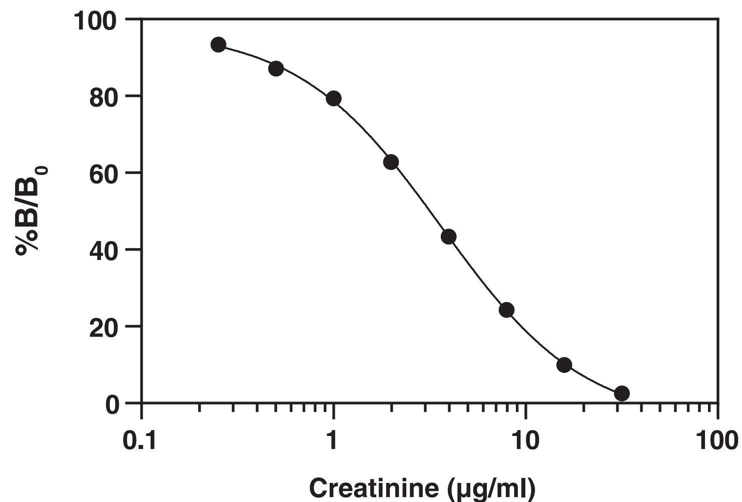
Representative Data

The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve. Do not use the data below to determine the values of your samples.

Absorbance at 450 nm (30 minutes)

Creatinine Standards (µg/ml) and Controls	Blk-subtracted Absorbance	NSB-corrected Absorbance	%B/B_0	%CV* Intra-assay Precision	%CV* Inter-assay Precision
NSB	0.003	--	--	--	--
B_0	0.739	0.736	--	--	--
TA	0.194	0.191	--	--	--
35	0.022	0.019	2.5	5.0	2.8
17.5	0.077	0.074	10.0	7.6	2.5
8.75	0.182	0.179	24.3	5.6	1.8
4.38	0.322	0.319	43.3	4.6	1.8
2.19	0.465	0.462	62.8	6.5	2.0
1.09	0.588	0.585	79.4	11.1	6.0
0.55	0.644	0.641	87.1	17.1	11.9
0.27	0.690	0.687	93.3	28.3**	15.9

Table 2. Typical results
*%CV represents the variation in concentration (not absorbance) as determined using a reference standard curve
**Evaluate data in this range with caution



Assay Range = 0.273-35 µg/ml
Sensitivity (defined as 80% B/B_0) = 1.009 µg/ml
Midpoint (defined as 50% B/B_0) = 3.486 µg/ml
Lower Limit of Detection (LLOD) = 0.270 µg/ml

The sensitivity and midpoint were derived from the standard curves shown above. The standard was diluted with Immunoassay Buffer C (1X).

Figure 7. Typical standard curve

Precision:

Intra-assay precision was determined by analyzing 24 replicates of three human urine controls in a single assay.

Matrix Control (µg/ml)	%CV
1,122	8.5
689	3.5
234	5.1

Table 3. Intra-assay precision

Inter-assay precision was determined by analyzing of three human urine controls in eight separate assays on different days.

Matrix Control (µg/ml)	%CV
1,428	13.0
778	16.9
239	11.6

Table 4. Inter-assay precision

Cross Reactivity:

Compound	Cross Reactivity
Creatinine	100%
Uric Acid	<0.2%*
Creatine Phosphate	0.12%
1-Methyl-4-imidazoleacetic Acid (MIAA)	0.095%
Creatine	0.068%
Cyclocreatine	0.041%
Histamine	0.017%
Tryptophan	<0.01%
Urea	<0.01%

Table 5. Cross reactivity of the Creatinine ELISA
*Lowest cross-reactivity that can be reported due to limited solubility of uric acid

RESOURCES

Troubleshooting

Problem	Possible Causes
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique
High NSB (>10% of B_0)	A. Poor washing B. Exposure of NSB wells to specific antibody
Very low B_0	A. Trace organic contaminants in the water source B. Plate requires additional development time
Low sensitivity (shift in dose response curve)	A. Standard is degraded or contaminated B. Dilution error in preparing standards
Analyses of two dilutions of a biological sample do not agree (*i.e.*, more than 20% difference)	Interfering substances are present
Low signal in the sample wells (below the range of the standard curve)	A. HRP inhibitors are present; ensure that the samples and buffers are free of HRP inhibitors, such as azide B. Sample requires further dilution
Only TA wells develop	A. Trace organic contaminants in the water source B. Tracer was not added to the wells

Procedure	Blk	TA	NSB	B_0	Standards/ Samples
Dilute and mix	Mix all reagents gently				
Add Immunassay Buffer C (1X)	--	--	100 µl	50 µl	--
Add Standards/Samples	--	--	--	--	50 µl
Add Creatinine-HRP Tracer	--	--	50 µl	50 µl	50 µl
Add Creatinine ELISA Monoclonal Antibody	--	--	--	50 µl	50 µl
Incubate	Seal the plate and incubate 2 hours at room temperature on an orbital shaker				
Wash	Aspirate wells and wash 5 x ~300 µl with Wash Buffer (1X)				
Add TMB Substrate Solution	175 µl				
TA - Add Tracer additionally diluted 1:20	--	5 µl	--	--	--
Develop	Seal the plate and incubate for 30 minutes at room temperature on an orbital shaker protected from light				
Do Not Wash. Add HRP Stop Solution	75 µl				
Read	Read absorbance at 450 nm				

Table 6. Assay summary

	A	B	C	D	E	F	G	H
12								
11								
10								
9								
8								
7								
6								
5								
4								
3								
2								
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References

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NOTES

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